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14. ABSTRACT

The three yeast UBL-UBA proteins, Rad23, Ddi1 and Dsk2 bind both ubiquitin and the proteasome. They are not essential for viability and some redundancy in terms of stabilization of ubiquitinated substrates has been shown, suggesting that they may have overlapping functions. Here we showed that Rad23 is indeed redundant with both Ddi1 and Dsk2 for cell cycle related roles. Surprisingly, Ddi1 and Dsk2 do not show any redundancy but the triple deletion shows an synthetic defect, suggesting that Rad23 has at least two different roles in cell cycle progression during G2/M. In addition, we found that these putative roles do not include a role in SPB duplication or spindle dynamics. In addition, we show that a tetra-ubiquitin chain is able to bind several UBL-UBA proteins at once, which might explain the redundancies observed, as well as suggesting that these multiple interactions might be relevant for efficient but regulated delivery of ubiquitinated substrates to the proteasome.

15. SUBJECT TERMS
Rad23, Ddi1, Dsk2, Ubiquitin-proteasome system, protein degradation, cell cycle, spindle pole body

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Introduction

Genetic instability and aneuploidy are hallmarks of and might initiate cancers. Failures in the Nucleotide Excision Repair (NER) pathway as well as chromosome misegregation due to centrosome abnormalities are two known causes of genetic instability and aneuploidy respectively, and have been found in most breast tumours (Latimer et al., 2003; Salisbury, 2001).

There are three UBL-UBA proteins in budding yeast: Ddi1, Dsk2 and Rad23. Rad23 and Dsk2 have been reported to be involved in duplication of the Spindle Pole Body (SPB), the yeast equivalent of mammalian centrosomes (Biggins et al., 1996). A role for Rad23 and Ddi1 in cell cycle control (Clarke et al., 2001) as well as their homo- and hetero-dimerization has also been reported (Bertolaet et al., 2001a). In addition, these proteins have been shown to bind to ubiquitin (Bertolaet et al., 2001b) and the proteasome, leading to the hypothesis that they shuttle ubiquitinated substrates to the proteasome for degradation (Hartmann-Petersen et al., 2003).

This proposal aimed to gain a better understanding of the role of UBL-UBA proteins in cell cycle control, SPB duplication and NER. We have shown that Rad23 and Ddi1 interact through their UBL/UBA domains and that several UBL-UBA proteins can bind to a common tetra-ubiquitin chain (Kang et al., 2006), a process that might influence the efficiency of substrate delivery to the proteasome and explain the basis of their redundancy.

In addition, by using strains deleted for the UBL-UBA proteins in different combinations we have observed overlapping roles for these proteins in cell cycle control (Diaz-Martinez et al., 2006). *RAD23* is redundant with *DDI1* and *DSK2*, but *DDI1* and *DSK2* are not redundant with each other and the triple deletion shows a synthetic effect, suggesting the existence of at least two roles for *RAD23* in cell cycle control. The *rad23*Δ*ddi1*Δ*dsk2*Δ triple deletion strain delays both in G2/M phase and in mid-anaphase at high temperatures with duplicated spindle pole bodies. Cell cycle progression in the triple deletion strain can only be partially rescued by a *rad23* allele lacking the c-terminal UBA domain, suggesting that *RAD23* requires its c-terminal UBA domain for full function. In addition to their ability to bind ubiquitin and the proteasome, the UBL-UBA proteins also share the ability to homodimerize. Rad23 and Dsk2 dimerization requires their UBL and/or UBA domains whereas Ddi1 dimerization does not. We also showed that Ddi1 homodimerization is necessary for its cell cycle related functions.

The UBA2 domain of Rad23 is necessary for its cell cycle functions

Since both UBA domains of Rad23 have been shown to cooperate in ubiquitin binding and substrate stabilization (Bertolaet et al., 2001b; Chen et al., 2001), we decided to test the role of UBA2 by looking at the cell cycle progression of a strain in which the solely UBL-UBA protein was *rad23ΔUBA2* (Figure 1). By looking at serial dilutions of the different strains, a partial recovery can be observed in the UBA2-deleted strain, but full recovery is only seen when the full-length RAD23 gene is present. To confirm this result in more detail, we looked at the kinetics of cell cycle progression of these strains after release from alpha-factor arrest at the non-permissive temperature (Figure 1B). G2/M accumulation

in the wildtype strain peaks at 120 minutes and decreases to 28% at 140 minutes after release. On the other hand, the triple deletion strain enters G2/M with the same kinetics as wildtype, but remains there even after 140 minutes (75% in G2/M). Meanwhile, ddi1Δdsk2Δrad23ΔUBA2 cells enter G2/M with the same kinetics as both wildtype and the triple deletion, but accumulate in G2/M, although at a lower level than the triple deletion (60% at 140 minutes), suggesting that complete function of Rad23 requires the full-length protein and that the UBA1-domain can only partially rescue the G2/M arrest.

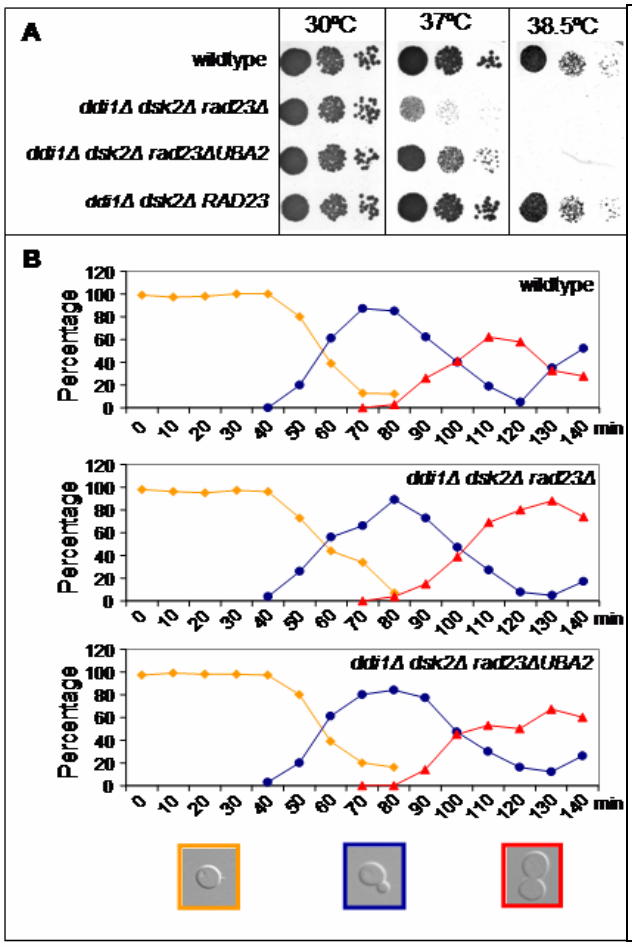

Figure 1. UBA2 domain of Rad23 is partially necessary for cell cycle progression
(A) Synthetic effect in temperature sensitivity. Cells were grown to mid-log phase and serial dilutions were spotted onto YEPD plates and incubated at the different temperatures for 48hrs.
(B) Kinetics of cell cycle progression in wildtype, *ddi1Δ dsk2Δ rad23Δ* and *ddi1Δ dsk2Δ rad23ΔUBA2* cells. Cells were arrested in G1 at 30ºC using 200ng/mL alpha-factor and released in rich medium at 37ºC. Cell cycle progression was monitored by bud morphology: unbudded cells (G1, yellow line), cells with small buds (S-phase, blue line) and cells with large buds (G2/M, red line).

Key accomplishments:

- Construction of more than 200 yeast strains with the different UBL-UBA genes deleted (*rad23Δ, dsk2Δ, ddi1Δ*) both singly and in combinations, harboring markers such as *SPC42:GFP* and/or *TUB1:GFP.*

- Evaluation of cell cycle progression in the different deletion strains, showing a partially redundant phenotype

- Evaluation of SPB duplication, showing no difference among wildtype, *dks2Δrad23Δ* and *ddi1Δdsk2Δrad23Δ*

- Design and integration of galactose-inducible vectors containing RAD23, DDI1 or DSK2 wildtype genes as well UBA and/or UBL deleted versions of these genes.

- Evaluation of the role of the UBL and/or UBA domains of these proteins in cell cycle progression and Spindle Pole Body duplication

- Construction and integration of different TAP-tagged version of RAD23, DDI1 and DSK2 for protein interaction analysis

- Construction of bacterial-expression plasmids harboring different domains of RAD23, DDI1 and DSK2 for NMR-analysis.

- Microscopic analysis of SPB duplication and mitotic spindle dynamics in strains lacking the different UBL-UBA proteins.

Reportable outcomes:

- Kang Y, Vossler R, **Diaz-Martinez LA**, Winter NS, Clarke DJ and Walters KJ (2006). UBL/UBA ubiquitin receptor proteins bind a common tetraubiquitin chain. *J Mol Biol* 356:1027-1035 (attached)

- **Díaz-Martínez LA**, Kang Y, Vossler R, Winter NS, Walters KJ and Clarke DJ (2006). Yeast UBL-UBA proteins have redundant functions in cell cycle control. *Cell Division* 1:28 (attached)

- **Díaz-Martínez LA.** PhD degree obtained. Thesis successfully defended on December 15th, 2006. Official date of degree completion: January 2006.

Conclusions

The three yeast UBL-UBA proteins bind both ubiquitin and the proteasome, suggesting that they may have overlapping functions. Here we showed that Rad23 is indeed redundant with both Ddi1 and Dsk2 for cell cycle related roles. Surprisingly, Ddi1 and Dsk2 do not show any redundancy but the triple deletion shows an additive defect, suggesting that Rad23 has at least two different roles in cell cycle progression during G2/M, one that is shared with Ddi and another one shared with Dsk2. In addition, we found that these putative roles do not include a role in SPB duplication or spindle dynamics durig mitosis. Furthermore, we show that a tetra-ubiquitin chain is able to bind several UBL-UBA proteins at once, which might explain the redundancies observed, as well as suggesting that these multiple interactions might be relevant for efficient but regulated delivery of ubiquitinated substrates to the proteasome.

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UBL/UBA Ubiquitin Receptor Proteins Bind a Common Tetraubiquitin Chain

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The ubiquitin–proteasome pathway is essential throughout the life cycle of a cell. This system employs an astounding number of proteins to ubiquitylate and to deliver protein substrates to the proteasome for their degradation. At the heart of this process is the large and growing family of ubiquitin receptor proteins. Within this family is an intensely studied group that contains both ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains: Rad23, Ddi1 and Dsk2. Although UBL/UBA family members are reported to regulate the degradation of other proteins, their individual roles in ubiquitin-mediated protein degradation has proven difficult to resolve due to their overlapping functional roles and interaction with each other and other ubiquitin family members. Here, we use a combination of NMR spectroscopy and molecular biology to reveal that Rad23 and Ddi1 interact with each other by using UBL/UBA domain interactions in a manner that does not preclude their interaction with ubiquitin. We demonstrate that UBL/UBA proteins can bind a common tetraubiquitin molecule and thereby provide strong evidence for a model in which chains adopt an opened structure to bind multiple receptor proteins. Altogether our results suggest a mechanism through which UBL/UBA proteins could protect chains from premature de-ubiquitylation and unnecessary elongation during their transit to the proteasome.

Keywords: Rad23; Ddi1; ubiquitin receptor proteins; proteasome-mediated protein degradation; ubiquitin-associated domains

Introduction

Ubiquitin signaling regulates an astounding array of cellular events and remains essential throughout the life cycle of a cell. In its most established role ubiquitylation targets proteins for degradation by the 26 S proteasome,[1] a process important for controlling the lifespan of regulatory proteins, removing misfolded proteins,[2] producing immunocompetent peptides,[3] activating and repressing transcription,[4,5] and regulating cell cycle progression.[6] In addition ubiquitylation can signal proteasome-independent events including endocytic sorting[7,8] and DNA repair.[9,10] Ubiquitylation is connected to proteasome-mediated protein degradation by an intricate network of ubiquitin recognition proteins. Elucidating this network remains a difficult albeit active area of research, as it is clouded by redundancy and cooperation between the large and growing ubiquitin receptor protein family. Among these proteins exists a group that harbors both ubiquitin-associated (UBA) and ubiquitin-like (UBL) domains (Figure 1(a)).

UBL/UBA proteins have attracted much attention for their ability to regulate the lifespans of other proteins. In *Saccharomyces cerevisiae*, Rad23 (hHR23a/b in humans), Dsk2 (hPLIC-1/2 in humans) and Ddi1 are UBL/UBA proteins that recruit ubiquitylated substrates to the proteasome for their degradation[11–16] *via* UBA domain interactions with ubiquitin[17–19] and UBL domain interactions with the proteasome.[20–23] Depending on their protein levels, UBL/UBA-containing proteins can also inhibit the degradation of ubiquitylated substrates.[16] Such inhibition occurs because UBA

Abbreviations used: Rad23, radiation-sensitive mutant 23; Ddi1, DNA damage-inducible protein; hHR23, human homolog of Rad23; HSQC, heteronuclear single quantum coherence; R4B, Rad4 binding domain; UBA, ubiquitin-associated; UBL, ubiquitin-like; GST, glutathione-*S*-transferase.

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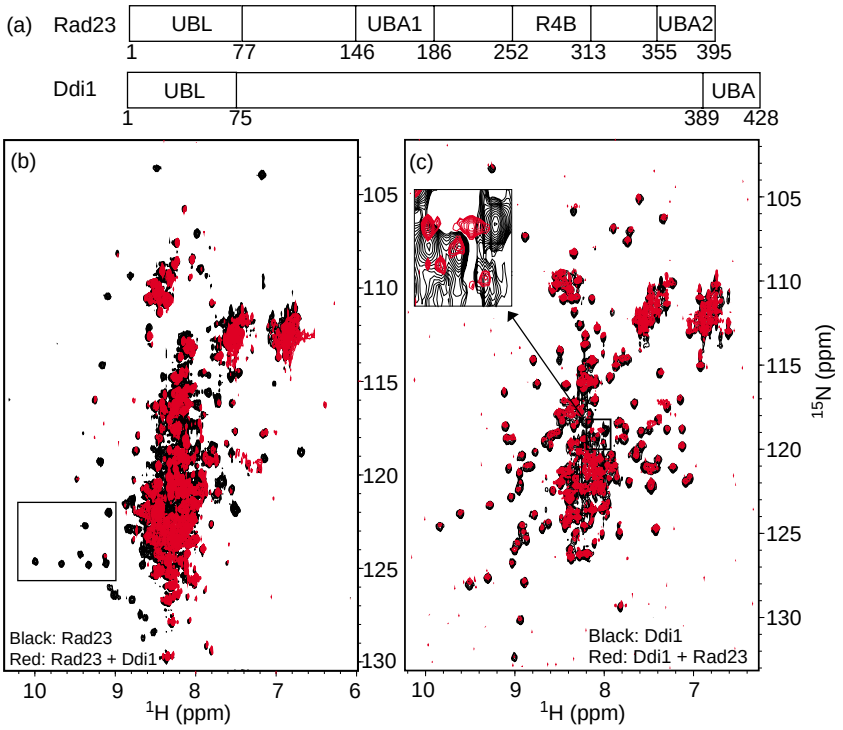

Figure 1. Ubiquitin recognition proteins Rad23 and Ddi1 interact. (a) The sequence location of Rad23's and Ddi1's UBL and UBA domains as well as Rad23's Rad4/XPC binding domain is illustrated. (b) Comparison of the [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Rad23 alone (black) to that acquired in the presence of fourfold molar excess Ddi1 reveals that Ddi1 causes severe broadening of certain Rad23 amide resonances (boxed in black). (c) [^{1}H,^{15}N] HSQC spectra are displayed of Ddi1 alone (red) and with fourfold molar excess Rad23 (red). The zoomed region highlights chemical shift perturbations in Ddi1's [^{1}H,^{15}N] HSQC spectrum caused by Rad23 addition. Together (b) and (c) provide strong evidence for the direct interaction of Rad23 with Ddi1.

domains sequester K48-linked polyubiquitin chains to in turn prevent their elongation and de-ubiquitylation.[16,24,25] In a perhaps related role, C-terminal UBA domains are reported to protect Rad23/hHR23a, Ddi1 and Dsk2 from their own degradation *via* the proteasome.[26]

Adding to the complexity of the ubiquitin family network, Rad23 is reported to interact with Ddi1 and Dsk2, and yeast two-hybrid experiments implicate UBA/UBA domain interactions as essential for such dimerization.[27,28] However, in previous work on hHR23a we found no such UBA/UBA domain interactions but instead found that the UBL domain of hHR23a interacts dynamically with each of its UBA domains.[29] Furthermore, whereas Rad23 is reported to dimerize,[18,27,28] hHR23a does not.[29]

To resolve these ambiguities we used NMR spectroscopy to determine the mechanism by which Rad23 binds Ddi1 and itself. Yeast two-hybrid experiments are unable to discriminate between direct interactions and those that are mediated by other proteins, both of which, as we report here, are available to Rad23 and Ddi1. Here, we reveal that UBL/UBA and not UBA/UBA domain interactions result in heterodimerization of Rad23 and Ddi1. These findings demonstrate the first published example for UBL/UBA domain interactions mediating heterodimerization. According to its crystal structure, the ubiquitin moieties of K48-linked tetraubiquitin are packed against each other with only the most distal moiety available for binding a UBA domain.[30] This structure suggests that K48-linked tetraubiquitin, which is the smallest chain length that signals for proteasome degradation[31] is able to bind only one ubiquitin receptor protein. In addition, Rad23 has two UBA domains, the C-terminal of which is reported to sandwich between the two ubiquitin subunits of diubiquitin.[32] Surprisingly, we have found that K48-linked tetraubiquitin can bind simultaneously to two Rad23 molecules as well as to Rad23 and Ddi1. This finding illustrates that K48-linked tetraubiquitin adopts an opened structure when bound to its receptors and leads to a working model for how ubiquitylated substrates are transferred to the proteasome.

Results

Rad23 heterodimerizes with Ddi1 *via* UBL/UBA domain contacts

Yeast two-hybrid experiments suggest that Rad23 interacts with itself, Dsk2 and Ddi1 by using UBA/UBA domain interactions.[27,28] These *in vivo* analyses, however, do not exclude the possibility of indirect associations through bridging molecules such as ubiquitin chains, which are known to interact with UBA domains.[18] To test whether Rad23 and Ddi1 interact in their purified forms we performed [^{1}H,^{15}N] heteronuclear single quantum coherence (HSQC) experiments on ^{15}N-labeled Rad23 or Ddi1 alone and in the presence of the other protein (Figure 1(b) and (c)). Such experiments detect amide nitrogen and proton atoms and their frequencies in spectra depend on their chemical environment; a phenomenon that makes them useful for detecting protein–protein interactions. The [^{1}H,^{15}N] HSQC spectrum of Rad23 resembles

that of its human homolog hHR23a, in that many sharp resonances appear in the region expected for randomly coiled residues (Figure 1(b)). This attribute originates from their long unstructured flexible linker regions that connect each of their small domains,[29] which for Rad23 comprise 42.7% of its amino acid residues.

In each set of [^{1}H,^{15}N] HSQC experiments direct interaction between Rad23 and Ddi1 was detected by spectral changes (Figure 1(b) and (c)). For Ddi1 the changes were confined to chemical shift perturbations (Figure 1(c)), whereas resonances derived from Rad23 experienced severe broadening and signal decay (Figure 1(b)). Although structural data and chemical shift assignments are available for the 40 kDa hHR23a protein,[29] neither Rad23 nor Ddi1 has been characterized structurally by NMR or X-ray crystallography.

Therefore, to identify the domains involved in forming the Rad23/Ddi1 protein complex we performed [^{1}H,^{15}N] HSQC experiments on single domain constructs of Rad23's UBL, UBA1 and UBA2 domains and on Ddi1's UBA domain. Comparisons between the truncated and full-length protein constructs allowed us to identify resonances derived from Rad23's UBL and UBA1 domains (Figure 2(a)) as well as Ddi1's UBA domain (data not shown). These resonance assignments were then used to interpret the results of the titration experiments recorded on the full-length proteins (Figure 1(b) and (c)). At equimolar protein concentration Rad23's UBL domain interacts with Ddi1's UBA domain and additional contacts are made between Ddi1's UBL domain and Rad23's internal UBA domain when Rad23 is present at twofold molar excess or greater (data now shown).

To test these UBL/UBA domain interactions more directly we performed analogous experiments in which we titrated the UBL domain of Rad23 into ^{15}N labeled full-length Ddi1 (Figure 3(a)) or its UBA domain (Figure 3(b)). We confirmed that the UBL domain of Rad23 binds the UBA domain of Ddi1 (Figure 3(b)), and found that the UBL domain of Rad23 induces identical chemical shift changes in Ddi1's [^{1}H,^{15}N] HSQC spectrum compared to equimolar concentrations of full-length Rad23 (Figure 3(a)). Finally, we tested directly whether either UBA domain of Rad23 can bind that of Ddi1 by performing [^{1}H,^{15}N] HSQC experiments on ^{15}N-labeled Ddi1 UBA domain alone and in the presence of either of Rad23's UBA domains. Even at eightfold molar excess Rad23's UBA domains do not cause chemical shift changes in that of Ddi1. Therefore, we conclude that Rad23 UBA domains do not interact with that of Ddi1 (data not shown).

Rad23 and Ddi1 dissociate to bind ubiquitin

Since Rad23 and Ddi1 bind ubiquitin with their UBA domains, we tested whether the Rad23/Ddi1 heterodimer remains intact in the presence of monoubiquitin. To ^{15}N-labeled Rad23 mixed with fourfold molar excess Ddi1 (Figure 4(a)), we added monoubiquitin such that the molar ratio of Rad23/Ddi1/monoubiquitin is equal to 1:4:10 (Figure 4(b)). At 1:4:10 molar ratio, the resonances derived from Rad23's UBL domain are restored, indicating that it is no longer bound to Ddi1's UBA domain. This loss of interaction is due to monoubiquitin successfully competing for Ddi1's UBA domain. In addition, resonances of Rad23's UBA1 and UBA2 domains shift upon ubiquitin addition, confirming that each

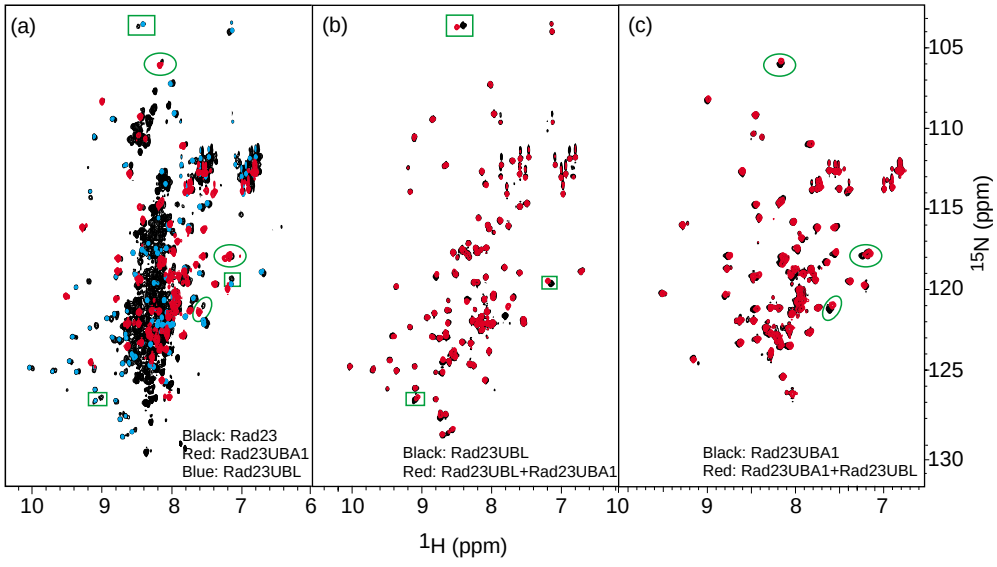

Figure 2. Rad23's UBA1 binds its own UBL domain. (a) The [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Rad23 (black) is superimposed onto that derived from single domain constructs of its UBA1 (red) and UBL (blue) domains. (b) Superimposed [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23's UBL domain alone (black) and at equimolar concentration with its UBA1 domain (red). (c) The converse experiment is shown: [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23's UBA1 domain alone (black) and with its UBL domain at equimolar ratio (red). Selected shifted UBL and UBA1 resonances are boxed by green rectangles and ovals, respectively.

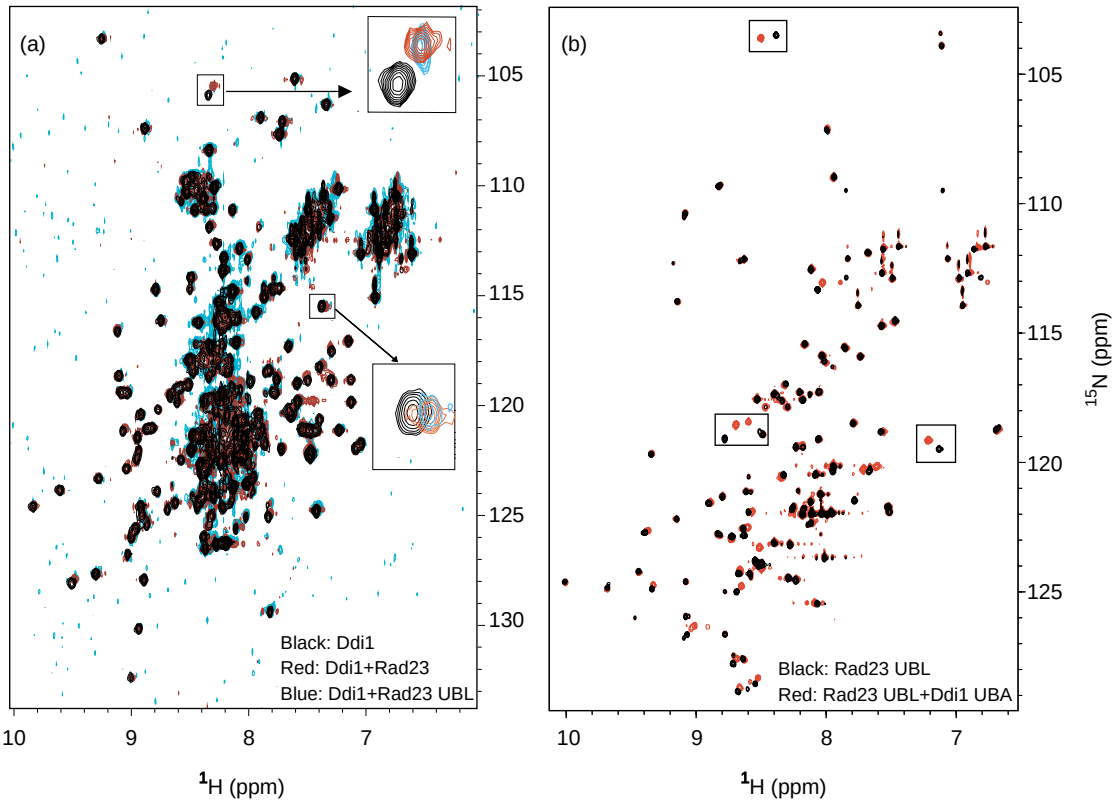

Figure 3. Rad23/Ddi1 heterodimerize *via* UBL/UBA domain interactions. (a) The [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Ddi1 alone (black) and with fourfold molar excess of either full-length Rad23 (red) or its UBL domain (blue). Rad23 and its UBL domain induce identical shifts in Ddi1's UBA domain. (b) The [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Rad23 UBL domain alone (black) and with Ddi1's UBA domain at equimolar concentration (red). Selected resonances of Rad23's UBL domain that shift by adding Ddi1's UBA domain are boxed.

of Rad23's UBA domains binds ubiquitin (Figure 4(b)).

Rad23 forms a homodimer through its C-terminal half

In addition to forming a heterodimer, Rad23 and Ddi1 each homodimerize. Ddi1 dimerization is independent of its UBL and UBA domain and occurs through residues located in the middle of its amino acid sequence.[27] These residues are absent from the [^{1}H,^{15}N] HSQC spectrum acquired on full-length Ddi1. Such absences are caused by line broadening due to chemical exchange or slow tumbling times (from bulkiness) and offer further support of an internal Ddi1 dimerization domain. That Ddi1's UBL and UBA domains are observable reflects their structural independence from its dimerization domain. [^{1}H,^{15}N] HSQC spectra reveal that the resonances of Ddi1's UBA domain superimpose well onto those derived from its full-length protein, as only two residues experience chemical shift changes due to this truncation (data not shown). These results support a model in which Ddi1's UBA and UBL domains are autonomous and connected to the rest of the protein by flexible linker regions.

In contrast to Ddi1, the mechanism by which Rad23 dimerizes is not well understood. Dynamic light-scattering experiments indicate that 99% of Rad23 species (at 23.6 μM) exist as a dimer in its purified form (data not shown). Interestingly, its human homolog hHR23a does not dimerize[29] and Rad23 dimerization was hypothesized to occur *via* UBA/UBA domain interactions.[27] In our [^{1}H,^{15}N] HSQC experiments chemical shift changes compared to full-length Rad23 were observed for its UBA1 domain when produced as a single domain construct (Figure 2(a)). Such changes suggest interactions with other regions of the protein and we performed titration experiments with single domain constructs to discover that it binds its own UBL domain (Figure 2(b) and (c)).

Most surprising, however, was that most of the resonances from the XPC/Rad4-binding and UBA2 domains were absent from the [^{1}H,^{15}N] HSQC spectrum recorded on full-length Rad23. These absences persisted even in an experiment performed for 14 h at 800 MHz with 128 increments in the ^{15}N dimension and 128 scans per increment on a ^{15}N-labeled 0.5 mM Rad23 sample. In contrast, all resonances derived from the XPC-binding and UBA2 domains are prominent in spectra recorded on hHR23a, even in experiments recorded with

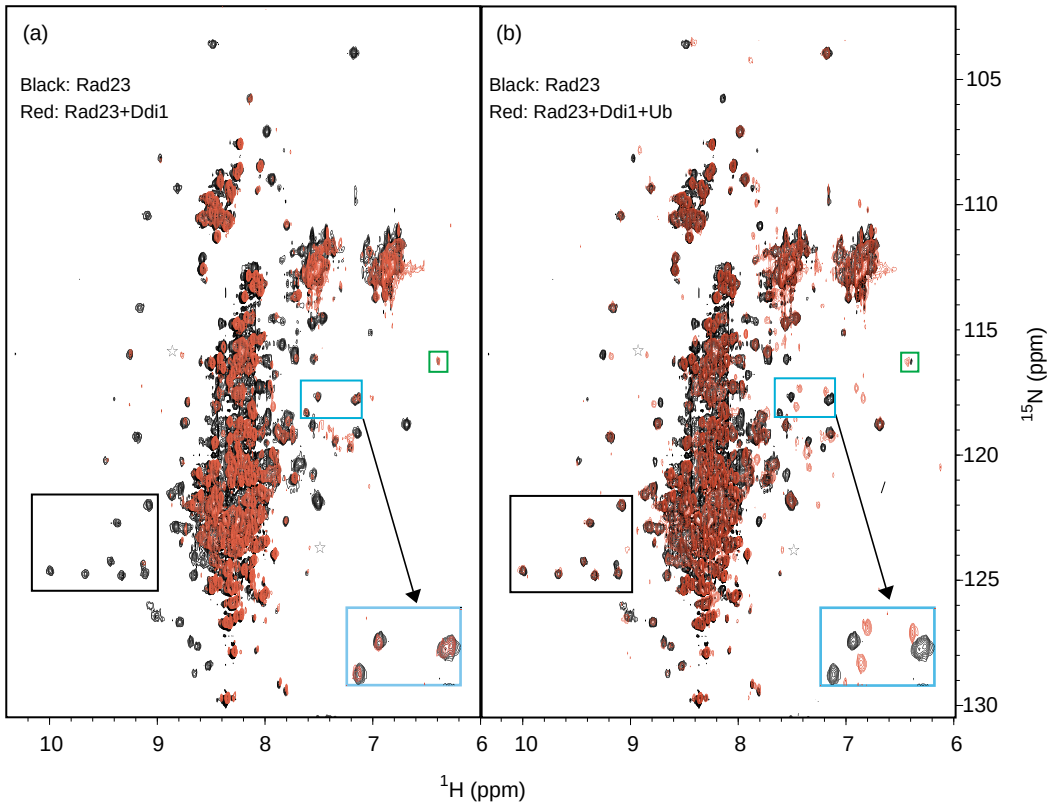

Figure 4. Rad23 and Ddi1 dissociate to bind ubiquitin. (a) [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23 alone (black) and with fourfold molar excess of Ddi1 (red) reveals that resonances of Rad23's UBL domain disappear upon binding Ddi1. (b) [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23 alone (black) and with Ddi1 and ubiquitin at 1:4:10 molar ratio, respectively, reveal ubiquitin to restore the broadened Rad23 resonances (boxed in black), and to cause chemical shift perturbations in Rad23's UBA1 (boxed in blue) or UBA2 (boxed in green) domains. Certain UBA2 resonances appear upon Ddi1 or ubiquitin addition (marked with an asterisk).

eight scans per increment and 0.1 mM sample concentration.[29] Interestingly, certain UBA2 resonances of Rad23 appear upon addition of Ddi1 (Figure 4(a)) or ubiquitin (Figure 4(b)); however, those of the Rad4-binding domain remain absent. These data support previous findings that Rad23 homodimerizes[18,27] and suggest a role for the Rad4-binding domain, which is consistent with the published finding that UBA2 is not sufficient for Rad23 dimerization.[28] The N-terminal half of Rad23 including its UBL and UBA1 domain does not appear to be in contact with its C-terminal half and they are most likely connected by a flexible linker region that allows them to move independently of one another, as the N-terminal but not the C-terminal half of Rad23 is observable.

Tetraubiquitin bridges ubiquitin receptor proteins

We were interested in how the ability of Rad23 and Ddi1 to form homo- and heterodimers impacts their polyubiquitin-binding mechanisms and in particular whether one tetraubiquitin molecule can bind more than one ubiquitin receptor protein. We therefore tested whether tetraubiquitin can bind more than one Rad23 or Ddi1 molecule. To test whether tetraubiquitin is capable of binding more than one Rad23 molecule we incubated K48-linked tetraubiquitin with Ni-NTA agarose resin containing pre-bound His-Rad23. After removing the unbound tetraubiquitin, glutathione-*S*-transferase (GST)-Rad23 was added and the beads washed again to remove unbound GST-Rad23. The resin-bound protein complex was fractionated by gel electrophoresis, transferred to a membrane, and probed with anti-GST or anti-ubiquitin antibody (Figure 5(a)). This experiment revealed the presence of a ternary complex containing GST-Rad23, His-Rad23 and tetraubiquitin. Since ubiquitin binding is reported to cause Rad23 homodimers to dissociate,[27] we hypothesized that this ternary complex must be formed by each Rad23 construct binding directly to tetraubiquitin. In addition, this experiment was performed at a temperature (4 °C) that does not permit monomer exchange in Rad23 homodimers as GST-Rad23 and His-Rad23 failed to interact in the absence of tetraubiquitin (Figure 5(a), lower panel, lane 4).

To test whether its human homolog hHR23a occupies all ubiquitin moieties of tetraubiquitin we examined whether the hHR23a–tetraubiquitin complex can also bind Rad23 (Figure 5(b) and (c)). Indeed, in experiments analogous to that described

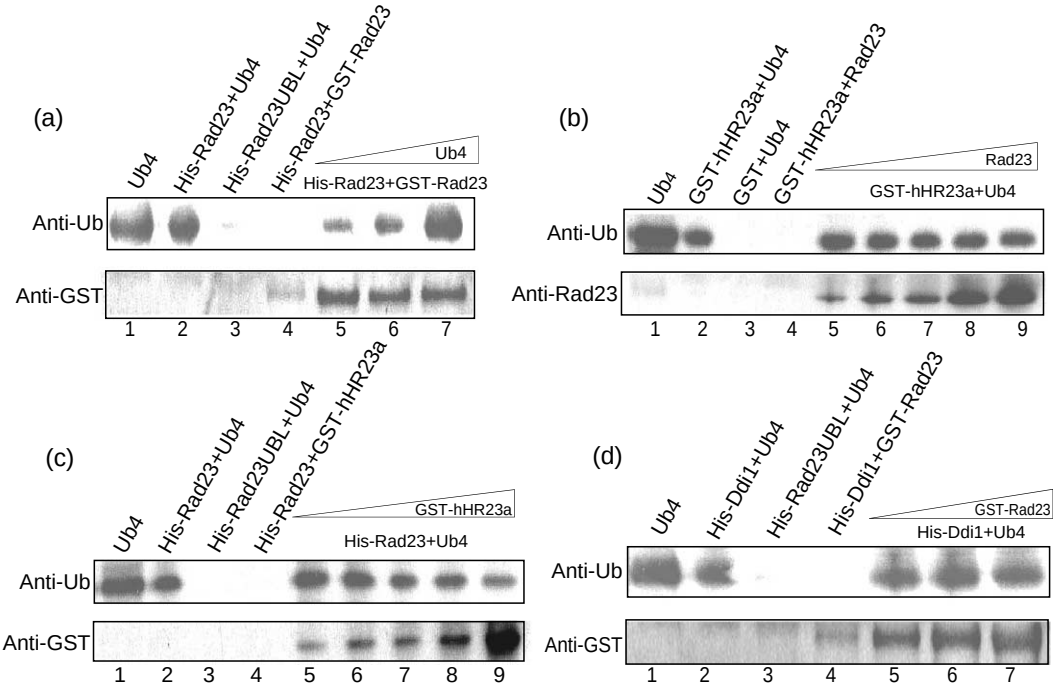

Figure 5. Ubiquitin receptor proteins Rad23 and Ddi1 bind a common tetraubiquitin molecule. In lanes 5–7 of (a), 20 μl of Ni-NTA resin pre-incubated with 0.1 nmol of His-Rad23 was mixed with 0.1 nmol of GST-Rad23 and increasing quantities of K48-linked tetraubiquitin, 0.008, 0.033, 0.132 nmol, respectively, and probed with anti-ubiquitin (top panel) and anti-GST (bottom panel). As a negative control, this experiment was performed on His-Rad23 UBL mixed with tetraubiquitin (lane 3), or in the absence of tetraubiquitin (lane 4). As a positive control, the experiment was performed without GST-Rad23 (lane 2). The 0.01 nmol of K48-linked tetraubiquitin (Boston Biochem.) used in these experiments and those in (b), (c) and (d) was loaded directly onto lane 1 of each gel. In lanes 5–9 of (b), 20 μl of glutathione S-Sepharose resin pre-incubated with 0.1 nmol of GST-hHR23a was mixed for 1 h at 4 °C with 0.033 nmol of tetraubiquitin and increasing quantities of untagged Rad23: 0.033, 0.067, 0.1, 0.2 and 0.4 nmol, respectively. After washing the resin the proteins were fractionated, transferred to a membrane and probed with anti-ubiquitin (top panel) or anti-Rad23 (lower panel). Indeed, the tetraubiquitin-Rad23-GST-hHR23a protein complex was observed. In lanes 5–9 of (c) the converse experiment was performed whereby 20 μl of Ni-NTA resin pre-incubated with 0.1 nmol of His-Rad23 was mixed for 1 h at 4 °C with 0.033 nmol of tetraubiquitin and increasing quantities of GST-hHR23a: 0.033, 0.067, 0.1, 0.2 and 0.4 nmol, respectively. The samples were treated as described for (a) and probed with anti-ubiquitin (top panel) or anti-GST (bottom panel). As a control, the analogous experiment was performed in the absence of tetraubiquitin to ensure that hHR23a and Rad23 do not interact directly (lane 4 of (b) and (c)). In addition, tetraubiquitin binding to GST-hHR23a (lane 2 of (b)) and His-Rad23 (lane 2 of (c)) but not to either tag or resin (lane 3 of (b) and (c)) was demonstrated. In lanes 5–7 of (d), 20 μl of Ni-NTA resin pre-incubated with 0.1 nmol of His-Ddi1 was mixed for 1 h at 4 °C with 0.132 nmol of tetraubiquitin and increasing quantities of GST-Rad23: 0.1, 0.5 and 1.0 nmol, respectively. The samples were treated as described above and probed with anti-ubiquitin (top panel) and anti-GST (bottom panel). Positive control with no GST-Rad23 (lane 2) and a negative control using His-Rad23 UBL with tetraubiquitin (lane 3) were also performed. Without tetraubiquitin, Rad23/Ddi1 interaction under these conditions is weak (lane 4, lower panel), whereas in the presence of tetraubiquitin, the Rad23/Ddi1/tetraubiquitin ternary complex forms (lanes 5–7).

above, tetraubiquitin was found to form a ternary, complex with Rad23 and hHR23a. Furthermore, the addition of hHR23a to resin-bound His-Rad23/tetraubiquitin reduced the amount of tetraubiquitin that was retained on the resin (Figure 5(c)), a trend that was not observed when Rad23 was added to resin-bound GST-hHR23a/tetraubiquitin (Figure 5(b)). This finding suggests that hHR23a binds tetraubiquitin more strongly than Rad23, which most likely stems from the loss of UBA2-mediated homodimerization.

Altogether our results support a model in which the Rad23 dimer dissociates upon binding tetraubiquitin and each molecule is used to bind individual moieties. In addition, these experiments suggest that although the hHR23a UBA2 domain sandwiches between the two ubiquitin moieties of diubiquitin,[32] the hHR23a/Rad23 UBA domains can also bind a single ubiquitin moiety of tetraubiquitin, as supported by the complexes that these UBA domains form with monoubiquitin.[19,33]

Analogously, since monoubiquitin is able to dissociate the Rad23/Ddi1 heterodimer, we hypothesized that these proteins could also form a ternary complex with tetraubiquitin. We therefore incubated tetraubiquitin with Ni-NTA agarose resin containing pre-bound His-Ddi1. After extensive washing, GST-Rad23 was added to the mixture, which was washed extensively again and probed for GST-Rad23 by performing Western blot analysis with anti-GST antibody (Figure 5(d)). This experiment supported our hypothesis that tetraubiquitin

can simultaneously bind two different ubiquitin receptor proteins as much more GST-Rad23 is retained on the resin in its presence.

Discussion

Perhaps the most impressive attribute of ubiquitin receptor proteins and polyubiquitin is their adaptability. The ubiquitin moieties of K48-linked tetra-[30] and diubiquitin[34] pack against each other to form a closed structure. Recent literature, however, suggests a flexible model for polyubiquitin structure,[35,36] and diubiquitin forms an open structure to bind the C-terminal UBA domain of hHR23a.[32] Here, we demonstrate that tetraubiquitin can bind more than one ubiquitin receptor protein and thereby reveal that it too opens to bind UBA domains. Our rationale for this conclusion is that only the most distal subunit relative to an attached protein substrate is available for binding a UBA domain in its closed structure.

The structural changes that occur in polyubiquitin parallel those of Rad23's human homolog hHR23a. In an earlier study we revealed that hHR23a forms a closed structure *via* UBL/UBA domain interactions that opens to bind either ubiquitin[19] or the proteasome component S5a.[29] Here, we demonstrate such UBL/UBA domain interactions to similarly mediate Rad23/Ddi1 heterodimerization and be displaced by ubiquitin.

Our findings invite the question: why do UBL/UBA ubiquitin receptor proteins interact? An answer to this question is suggested by two of the results presented here: the Rad23/Ddi1 heterodimer dissociates to bind polyubiquitin and these two proteins can bind a common polyubiquitin chain. Such attributes enable Rad23/Ddi1 heterodimers to bind a greater number of ubiquitin moieties in a polyubiquitin chain than either protein alone. This feature is important because Rad23 binding to polyubiquitin can block its elongation[16,24,25] and de-ubiquitylation,[25] most likely by precluding chains from the enzymes required for these activities. We therefore propose that dimerization of UBL/UBA domain proteins plays an important role in preventing unnecessary and therefore wasteful chain elongation or premature chain disassembly during the transit of ubiquitylated substrates to the proteasome (Figure 6).

In summary, we reveal that UBL/UBA ubiquitin receptor proteins interact with each other in a manner that does not interfere with their binding to polyubiquitin. On the contrary, since these proteins bind a common polyubiquitin chain, we propose such heterodimerization to aid in their ability to bind a greater number of ubiquitin moieties, an attribute that has implications for the chemistry performed on chains during their transit to the proteasome. In humans, Rad23 can bind an additional ubiquitin receptor protein, S5a,[20] which contains two ubiquitin interaction motifs (UIMs) rather than UBA domains. The S5a homolog in yeast (Rpn10) is truncated and lacks the C-terminal UIM to which hHR23a binds.[20] Consequently, it does not bind Rad23 and this example of an evolved interaction between two abundant ubiquitin receptor proteins offers further evidence for the importance of their collaborative relationships.

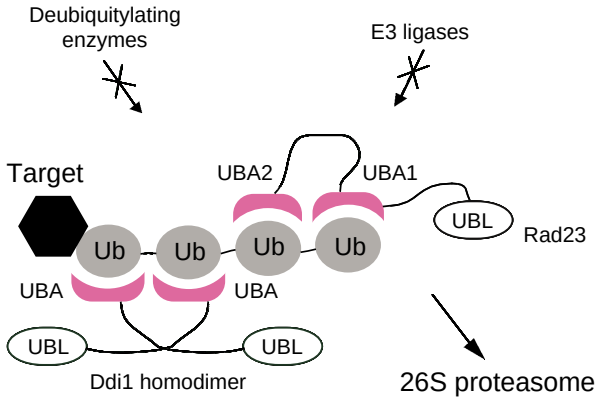

Figure 6. Proposed model for how UBL/UBA domain proteins prevent de-ubiquitylation and unnecessary chain elongation during the transit of substrates to the proteasome. A polyubiquitin chain is greeted with a complex containing multiple UBA domains that are available for binding individual moieties.

Materials and Methods

Sample preparation

For NMR spectroscopy Rad23, Ddi1 and single-domain constructs of the Rad23 UBL, UBA1 and UBA2 domains as well as of Ddi1's UBA domain were each cloned into the pET15b expression vector (Novagen) in-frame with the N-terminal histidine tag. The plasmids containing these genes were each transformed into *Escherichia coli* BL21 (DE3) cells and grown at 37 °C in M9 minimal medium or in Luria broth containing ampicillin (100 μg/ml). The cells were harvested 3 h after protein expression was induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG). The proteins were purified by using affinity purification on Ni-NTA resin as described.[21] Further purification was achieved on an FPLC system (Pharmacia), by either Superdex 200 (for full-length Rad23 and Ddi1) or 75 (for UBL and UBA domains) preparative columns. We produced ^{15}N-labeled samples for NMR spectroscopy by growth and expression in M9 minimal medium with [^{15}N]NH_4Cl as the only source of nitrogen. Unlabeled monoubiquitin was purchased (Sigma-Aldrich).

NMR spectroscopy

All NMR samples were dissolved in 20 mM $NaPO_4$ (pH 6.5), 100 mM NaCl, 0.1% (w/v) NaN_3, and 10% ^2H_2O. Spectra were acquired at 25 °C on Varian NMR spectrometers operating at either 800 MHz or 600 MHz. Processing was performed in NMRPipe[37] and the resulting spectra were visualized in XEASY.[38] Protein concentrations were calculated by using extinction coefficients based on amino acid composition and absorbance at 280 nm for protein dissolved in 8 M urea.

Western blot analysis

GST-tagged Rad23 and hHR23a were produced from the pGEX-2T and pGEX-6P-1 expression vectors (Amersham Pharmacia), respectively, by cloning their cDNA in-frame with glutathione-S-transferase (GST). Each protein was expressed and purified as described[29] and Rad23 was separated from GST by cleaving with thrombin. The 0.1 nmol of purified His-tagged Rad23 or Ddi1 or of purified GST-tagged hHR23a was bound to 20 μl of pre-washed Ni-NTA resin or glutathione S-Sepharose resin, respectively. Each resin was allowed to mix at 4 °C overnight with K48-linked tetraubiquitin (Boston Biochem Inc.) and then washed extensively with buffer A (20 mM sodium phosphate (pH 6.5), 100 mM NaCl, 0.5% (v/v) Triton X-100). The Ni-NTA resins were then incubated with either GST-Rad23 or GST-hHR23a for 1 h at 4 °C as the glutathione S-Sepharose resin was mixed with untagged Rad23 under the same conditions. Each resin was pelleted and then washed extensively with buffer A or buffer B (50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol). Proteins that were retained on the resin were fractioned by electrophoresis, transferred to a PVDF membrane, and probed with a polyclonal anti-ubiquitin (Boston Biochem Inc.), anti-Rad23,[18] or anti-GST (Santa Cruz Biotechnology) antibody. Visualization was performed using anti-rabbit-horseradish peroxidase and ECL.

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Yeast UBL-UBA proteins have partially redundant functions in cell cycle control

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Abstract

Background: Proteins containing ubiquitin-like (UBL) and ubiquitin associated (UBA) domains have been suggested to shuttle ubiquitinated substrates to the proteasome for degradation. There are three UBL-UBA containing proteins in budding yeast: Ddi1, Dsk2 and Rad23, which have been demonstrated to play regulatory roles in targeting ubiquitinated substrates to the proteasome for degradation. An involvement of these proteins in cell cycle related events has also been reported. We tested whether these three proteins act redundantly in the cell cycle.

Results: Here we show that the UBL-UBA proteins are partially redundant for cell cycle related roles. *RAD23* is redundant with *DDI1* and *DSK2*, but *DDI1* and *DSK2* are not redundant with each other and the triple deletion shows a synthetic effect, suggesting the existence of at least two roles for *RAD23* in cell cycle control. The *rad23Δddi1Δdsk2Δ* triple deletion strain delays both in G2/M-phase and in mid-anaphase at high temperatures with duplicated spindle pole bodies. Cell cycle progression in the triple deletion strain can only be partially rescued by a *rad23* allele lacking the c-terminal UBA domain, suggesting that *RAD23* requires its c-terminal UBA domain for full function. In addition to their ability to bind ubiquitin and the proteasome, the UBL-UBA proteins also share the ability to homodimerize. Rad23 and Dsk2 dimerization requires their UBL and/or UBA domains whereas Ddi1 dimerization does not. Here we show that Ddi1 homodimerization is necessary for its cell cycle related functions.

Conclusion: The three yeast UBL-UBA proteins have partially redundant roles required for progression through mitosis.

Background

The ubiquitin-proteasome pathway is a complex protein degradation system that is conserved from yeast to mammals and plays an important role in many processes such as cell cycle control, endocytosis and DNA repair [1-5]. In *Saccharomyces cerevisiae* Rad23, Ddi1 and Dsk2 are the three UBL-UBA proteins, which are hypothesized to shuttle ubiquitinated substrates to the proteasome for degradation [6-11] due to their ability to interact with the proteasome through their UBL domains [12-15] as well as with ubiquitin and polyubiquitinated substrates through their UBA domains [7,8,16-18]. Consistent with the shut-

tling hypothesis, downregulation of human *RAD23* (hHR23) using siRNA induces accumulation of p53 which is known to be continuously degraded by the ubiquitin-proteasome pathway [19]. In addition, yeast Rad23 is sufficient for docking of ubiquitin conjugates to proteasomes isolated from a strain carrying a mutation in the ubiquitin-interacting-motif (UIM) of the proteasome subunit Rpn10 [20], which is thought to act as a receptor that binds ubiquitinated substrates. Furthermore, proteasomes isolated from a *rad23Δdsk2Δ* strain are defective in their association with endogenous ubiquitin conjugates and this defect is stronger than the one observed in *rpn10* mutants that are unable to bind ubiquitin, suggesting that the UBL-UBA proteins are more active than Rpn10 in delivery of ubiquitinated substrates to the proteasome [20].

Interestingly, none of the UBL-UBA proteins are essential for viability. Single deletion of *RAD23* or *DSK2* induces partial stabilization of a model degradation-substrate whereas the *rad23Δdsk2Δ* double deletion completely stabilizes the substrate [7] and accumulates endogenous polyubiquitinated proteins at high temperatures [21], suggesting that Rad23 and Dsk2 function redundantly in ubiquitin-mediated protein degradation. In addition to these genetic interactions, UBL-UBA proteins have been shown to physically interact, forming both homodimers and heterodimers [7,22]. Dsk2 and Rad23 homodimerization occurs through their UBL and/or UBA domains [17,23,24], whereas Ddi1 homodimerization requires neither of these domains [17]. Although, in vivo, heterodimerization of UBL-UBA proteins is likely to also occur via bridging molecules [22], direct interactions between the UBL and UBA domains promoting heterodimer formation have been demonstrated [22,23]. Rad23 homo and heterodimerization of UBL-UBA family members has been suggested to play a role in regulating their interactions with other components of the ubiquitin-proteasome pathway and to lead to the formation of multimeric complexes with polyubiquitin chains [23,25]. Such interactions could increase the targeting efficiency of ubiquitinated substrates for degradation.

One of the cellular processes that relies on ubiquitin-proteasome dependent proteolysis is cell cycle progression. Cell division is a complex process; it requires that a series of steps are fulfilled in a specific and unidirectional order. When cell cycle regulation fails genetic instability and aneuploidy often arise, which are hallmarks of and might initiate cancers [26,27]. A long list of cell cycle regulators are known to be degraded by the ubiquitin-proteasome pathway [2]. Several pieces of evidence suggest a role for UBL-UBA containing proteins in cell cycle control. In budding yeast, overexpression of Dsk2 is toxic, inducing accumulation of ubiquitinated substrates [12] and arresting the cells in mitosis with abnormal nuclear position and short bipolar spindles [28]. Ddi1 is involved in the degradation of an SCF component, the F-box protein Ufo1, involved in the G1/S transition [29], as well as one of its targets the Ho endonuclease [30]. Overexpression of *DDI1* or *RAD23* suppresses the temperature sensitive phenotype of a *PDS1* mutant allele (*pds1–128*) [31]. Combined deletion of the *RAD23* and *DDI1* C-terminal UBAs, but not the single deletions, results in premature loss of cohesion and spindle elongation in the presence of hydroxyurea (HU), which is known to activate the S-phase checkpoint [31]. In addition, the *dsk2Δrad23Δ* double deletion, but not the single deletions, is reported to be defective in Spindle Pole Body (SPB) duplication at high temperature, inducing the formation of monopolar spindles and subsequently arresting the cells in mitosis [28]. These results suggest redundant roles for the UBL-UBA proteins in cell cycle events.

Here we demonstrate that deleting *RAD23* in combination with either *DDI1* or *DSK2* induces cell cycle delays in the G2/M-phase and anaphase at high temperatures, indicating that *DDI1* and *DSK2* are redundant with *RAD23*. In addition, we provide evidence that *DDI1* and *DSK2* are not functionally redundant and therefore that *RAD23* has at least two cell cycle-related functions. This hypothesis is supported by the synthetic effect observed in the triple deletion, confirming that *RAD23-DDI1* redundancy is different from that of *RAD23-DSK2* (i.e. the triple deletion shows an additive phenotype). Surprisingly however, the arrest is not due to failure in SPB duplication.

Results

RAD23, DDI1 *and* DSK2 *have partially redundant roles in cell cycle progression*

Possible roles for Rad23 and Ddi1 in the cell cycle have been suggested by their ability to rescue the temperature sensitivity of *pds1–128* [31], while Rad23 and Dsk2 have been reported to have roles in SPB duplication and Dsk2 overexpression leads to mitotic arrest [28]. To test whether the three yeast UBL-UBA proteins have a redundant role in cell cycle progression we took a genetic approach, obtaining strains deleted for each one of the UBL-UBA genes (Table 1) as well as all the double deletion combinations and the triple deletion, and asked whether any redundancy in terms of functions in cell cycle progression could be observed.

Although none of the single deletions have temperature sensitive phenotypes, the *rad23Δdsk2Δ* double deletion has been reported to accumulate ubiquitinated substrates at 37°C [21], as well as have a temperature sensitivity phenotype at 35°C [28]. Partially consistent with these results, we observed that *rad23Δ*, *ddi1Δ* or *dsk2Δ* singly deleted strains as well as *rad23Δddi1Δ* and *ddi1Δdsk2Δ*

Table 1: Yeast strains used in this study

1167	*MATalpha pds1–128*
1494	*pds1–128 GAL-ddi1ΔUBA [HIS]*
1493	*pds1–128 GAL-DDI1 [HIS]*
1671	*MATa bar1Δ GFP:TUB1::uRA3 ARG4*
2073	*MATalpha pds1–128 GAL-ddi1-A407L*
2074	*MATalpha pds1–128 GAL-ddi1-L426A*
L153	*MATalpha pds1–128 arg 4 GAL:ddi1Δ184–285*
L156	*MATalpha pds1–128 arg 4 GAL:ddi1Δ184–285*
L150	*MATalpha pds1–128 arg 4 GAL:DDI1 [LEU]*
L169	*MATa bar1Δ spc42:GFP [TRP]*
L182	*MATa bar1Δ dsk2::KAN spc42:GFP [TRP]*
L185	*MATa bar1Δ rad23::KAN dsk2::KAN SPC42:GFP [TRP]*
L187	*MATa bar1Δ ddi1::KAN dsk2::KAN SPC42:GFP [TRP]*
L194	*MATa bar1Δ rad23::KAN SPC42:GFP [TRP]*
L196	*MATa bar1Δ ddi1::KAN rad23::KAN SPC42:GFP [TRP]*
L201	*MATa bar1Δ ddi1::KAN rad23::KAN dsk2::KAN TUB1:GFP [URA]*
L218	*MATa bar1Δ ddi1::KAN dsk2::KAN rad23::KAN SPC42:GFP [TRP]*
L221	*MATa bar1Δ ddi1::KAN SPC42:GFP [TRP]*
L263	*MATa bar1Δ ddi1::KAN dsk2::KAN TUB1:GFP::URA3 RAD23ΔUBA2-MYC [TRP] SPC42:GFP [TRP]*

doubles were not temperature sensitive. Surprisingly, the *rad23Δdsk2Δ* double deletion was also alive at 37°C. In contrast, the *rad23Δdsk2Δddi1Δ* triple deletion was temperature sensitive at 37°C, suggesting the existence of redundant roles for Ddi1, Dsk2 and Rad23 at high temperatures.

Next we asked whether these proteins were involved in cell cycle progression. Briefly, all the combinations of single, double and the triple deletions were incubated in YEPD overnight at 30°C, diluted the next morning and incubated at 37°C. Cell cycle progression was evaluated by scoring the proportions of the different cell morphologies: unbudded cells (G1, yellow), cells with small to medium buds (S phase, blue) and dumbbells (G2/M, red) (Figure 1, upper panel). Samples were also taken for FACScan analysis of DNA content (Figure 1, lower panel). Interestingly both *rad23Δdsk2Δ* and *rad23Δddi1Δ* showed a slight accumulation of cells in G2/M, but the accumulation observed in the *rad23Δddi1Δdsk2Δ* triple deletion reached ~70% after 6 hours at the non-permissive temperature (Figure 1). On the other hand, cell cycle distribution in the *ddi1Δdsk2Δ* double deletion was not different from those of the single deletions. These results suggest that *RAD23* is redundant with *DDI1* and *DSK2* but that *DDI1* and *DSK2* do not share a common functional role needed for timely cell cycle progression. Furthermore, these data suggest a synthetic effect, that is, *RAD23* redundancy with *DDI1* is different than that of *RAD23* with *DSK2* and the effect seen in the triple deletion is the result of both defects combined (Figure 1).

The c-terminal UBA domain of Rad23 is necessary for its cell cycle functions

Although both UBA domains of Rad23 have been shown to bind ubiquitin both in yeast [17] and in humans (hHR23A) [32], there is a specific requirement for its c-terminal UBA domain (UBA2) in mediating cell cycle arrest after binding to the HIV-1 Vpr protein [33]. Therefore we tested whether UBA2 is required for the cell cycle function of Rad23 by examining cell cycle progression in a strain in which the sole UBL-UBA protein is *rad23ΔUBA2* (Figure 2). A recent study suggested that the C-terminal UBA domain of Rad23 acts as a stabilization domain [34], preventing proteasome-dependent degradation of RAd23. It was therefore important to determine if *rad23ΔUBA2* was stable in our strains. As shown in Figure 2A, the protein levels of *rad23ΔUBA2* and Rad23 were identical, whether these genes were expressed from their endogenous promoters or exogenously from the *GAL1* promoter. This was also the case when we compared the protein levels of Ddi1 and *ddi1ΔUBA* (Figure 2A). After confirming that the wild type and mutant proteins were present in identical amounts in our strains, we spotted serial dilutions of the corresponding strains onto rich medium and grew at a range of temperatures for several days (Figure 2B). A partial recovery of the temperature sensitivity of the *rad23Δddi1Δdsk2Δ* triple deletion strain was observed after introducing *rad23ΔUBA2*, but full recovery was observed only when the full-length *RAD23* gene was introduced (Figure 2B). These data suggest that, although both UBA-motifs can interact with ubiquitin [17], full Rad23 activity requires both UBA domains.

To explore this result in more detail, we examined the kinetics of cell cycle progression in strains that contained

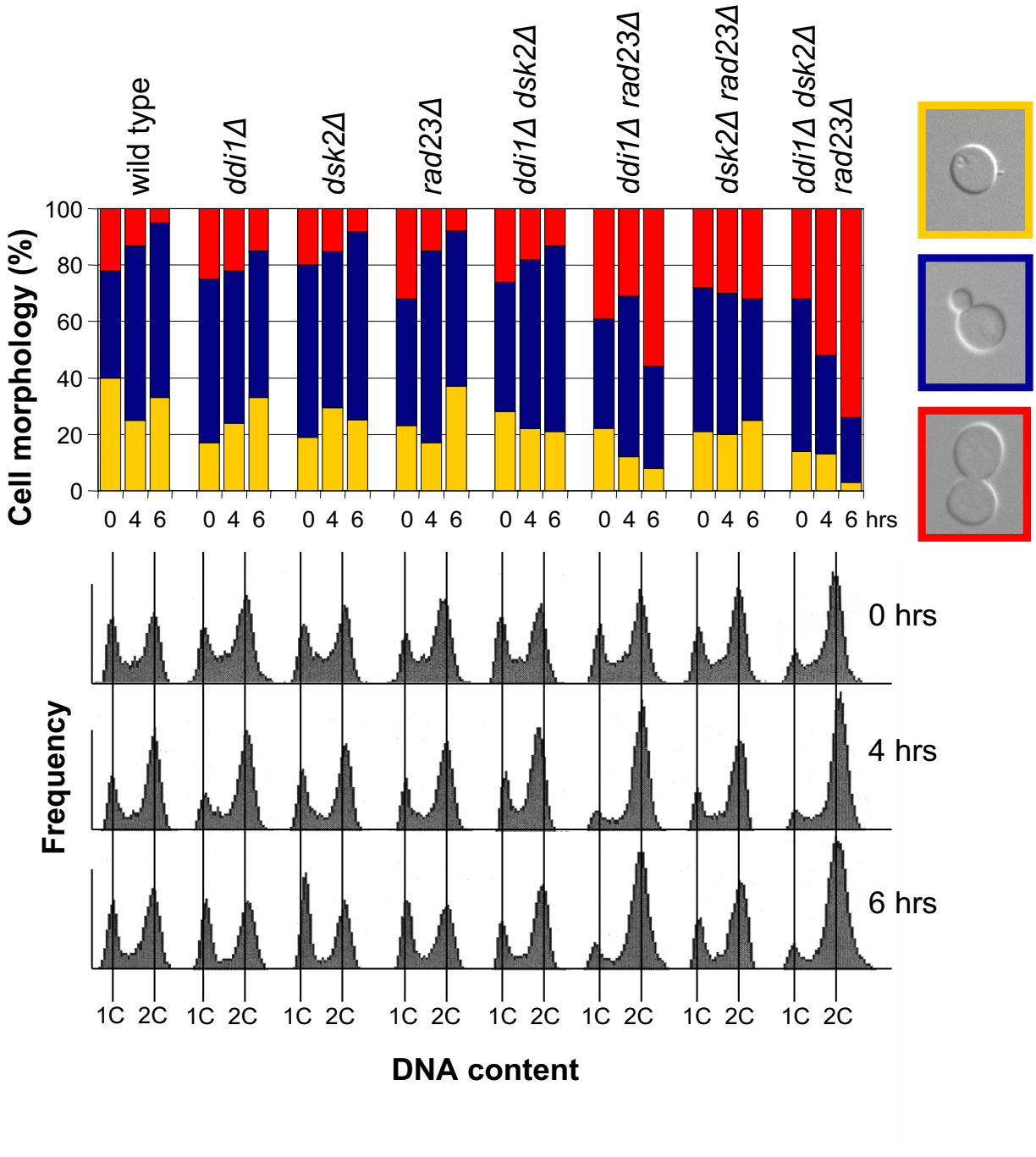

Figure 1

Deletion of *DDI1*, *DSK2* and *RAD23* has a synthetic effect on cell cycle progression at high temperatures. (A) Cells were grown to mid-log phase in liquid YEPD at 30°C then shifted to 37°C for 0, 4 and 6 hrs. Cell cycle distribution was determined by bud morphology: cells in G1 are unbudded (yellow), S-phase cells with small buds (blue) and G2/M cells with large buds (red). Large buds (dumbbells) are defined as cells where the bud is as big as the mother cell. In parallel, samples were taken for FACScan analysis of DNA content (lower panel).

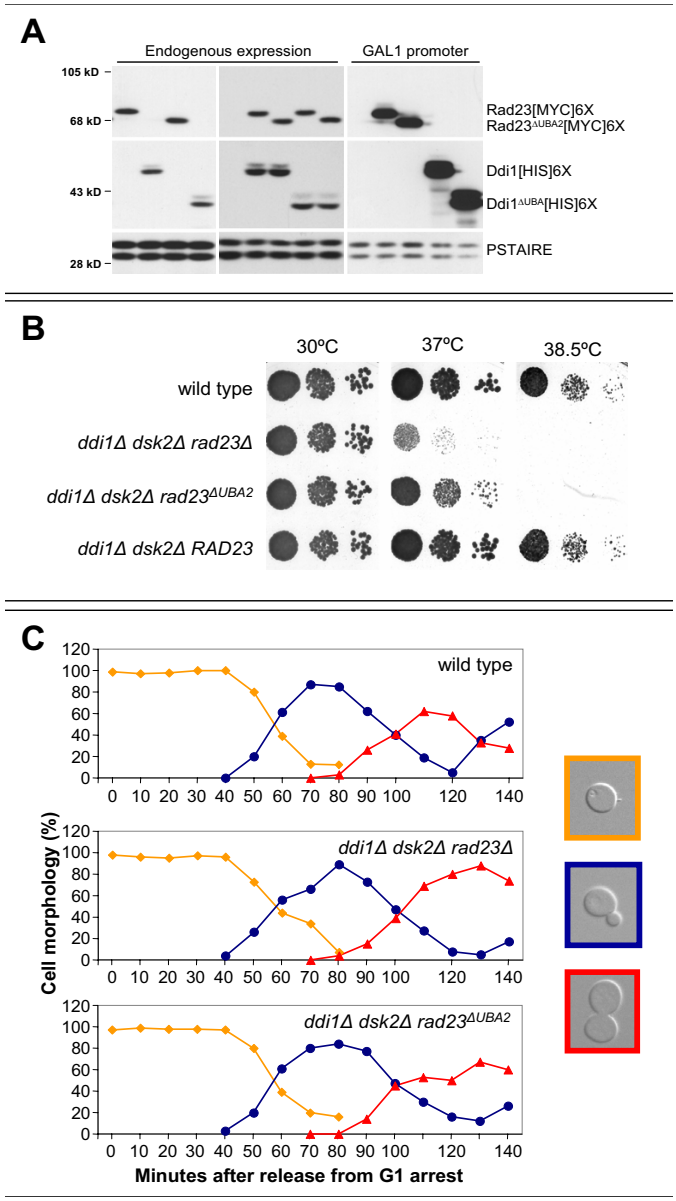

Figure 2

Cell cycle arrest in cells lacking *DDI1*, *DSK2* and the c-terminal UBA (UBA2) domain of Rad23. (A) Western blots showing relative protein levels of Rad23, Ddi1 and mutant forms in yeast extracts. Left two panels show endogenous levels of these proteins and the right panel shows levels produced after expression of constructs from the *GAL1* promoter. In each case, the wild type and mutant version were tagged identically at their C-termini – [MYC]6x for Rad23 and [HIS]6x for Ddi1. PSTAIRE is a loading control. Strains expressed (from left to right) – Left panel: *RAD23 [MYC]6x*, *DDI1 [HIS]6x*, *RAD23*$^{\Delta UBA2}$*[MYC]6x*, *DDI1*$^{\Delta UBA}$*[HIS]6x*; Middle Panel: no tag control, *RAD23[MYC]6x* and *DDI1[HIS]6x*, *RAD23*$^{\Delta UBA2}$*[MYC]6x* and *DDI1[HIS]6x*, *RAD23[MYC]6x* and *DDI1*$^{\Delta UBA}$*[HIS]6x*, *RAD23*$^{\Delta UBA2}$*[MYC]6x* and *DDI1*$^{\Delta UBA}$*[HIS]6x*; Right Panel: no tag control, *GAL1-RAD23[MYC]6x*, *GAL1-RAD23*$^{\Delta UBA2}$*[MYC]6x*, *GAL1-DDI1[HIS]6x*, *GAL1-DDI1*$^{\Delta UBA}$*[HIS]6x*. Upper band of Ddi1 is a phosphorylated species (data not shown). (B) Synthetic effect of UBL-UBA mutants on temperature sensitivity. The indicated strains were grown to mid-log phase and serial dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 48 hrs. Levels of Rad23 and Rad23$^{\Delta UBA2}$ protein expression were determined as shown in A. (C) Kinetics of cell cycle progression in wild type , *ddi1Δ dsk2Δ rad23Δ* and *ddi1Δ dsk2Δ rad23ΔUBA2* cells. (*ddi1Δdsk2Δ* strains behaved identically to the wild type control; Figure 1 and data not shown.) Cells were arrested in G1 at 30°C with alpha-factor and released in rich medium at 37°C. Cell cycle progression was monitored by bud morphology: unbudded cells (G1, yellow line), cells with small buds (S-phase, blue line) and cells with large buds (G2/M, red line).

either no UBL-UBA proteins (*rad23Δddi1Δdsk2Δ*) or *rad23ΔUBA2* (*ddi1Δdsk2Δrad23ΔUBA2*) as the sole UBL-UBA protein (Figure 2C). After release from a G1 arrest induced by alpha-factor treatment at the non-permissive temperature of 37°C, re-entry into the cell cycle and budding occurred with the same kinetics in these strains and in a wild type control. G2/M accumulation (red line) in the wild type strain peaked at 120 minutes and decreased to 28% 140 minutes after release as the cells entered the next cell cycle. Initiation of a second cell cycle was also indicated by the presence of a second wave of small-budded cells (blue line) appearing at 130 minutes. In contrast, the triple deletion strain entered G2/M with the same kinetics as wild type, but remained there even after 140 minutes (75% in G2/M). Meanwhile, *ddi1Δ dsk2Δ rad23ΔUBA2* cells entered G2/M with the same kinetics as both the wild type and the triple deletion strains, but accumulated in G2/M, albeit to a lesser extent than the triple deletion (60% at 140 minutes). This finding suggests that *rad23ΔUBA2* partially alleviates the delay and confirms that complete function of Rad23 requires the full-length protein and that the internal UBA domain (UBA1) can only partially alleviate the G2/M delay.

G2/M arrest is not due to a failure in SPB duplication

Since defects in SPB duplication leading to G2/M arrest have been reported for strains lacking *RAD23* and *DSK2* [28], we asked if there is a SPB duplication defect in the triple deletion strain. To visualize SPBs in live cells we used strains expressing *SPC42:GFP* (Table 1). Wild type or *rad23Δddi1Δdsk2Δ* triple deletion strains expressing *SPC42:GFP* were arrested in G1 using alpha-factor and released into fresh medium at the restrictive temperature. Samples were taken every 10 minutes, visualized (Figure 3A–B) and scored according to SPB number (Figure 3C). Surprisingly, the triple deletion strain separated SPBs normally (Figure 3A–C). The kinetics of SPB separation in the triple deletion were indistinguishable from that of the wild type strain (Figure 3D), except that the triple deleted cells arrested as large budded cells with two SPBs (Figure 3E,D). In the wild type strain, SPB separation initiated at ~40 minutes after release from G1 at the restrictive temperature (Figure 3C, yellow line) and peaked at ~80 minutes. In accordance, the population of cells with a single SPB signal (Figure 3C, blue line) was depleted within the same timeframe. After 100 minutes a second wave of cells with a single SPB signal peaked, followed by a second wave of SPB separation as cells enter another cell cycle. A mixed population of unbudded cells with single SPB signals and re-budded cells with newly separated SPBs could be observed 110 minutes after the alpha-factor release (Figure 3A, arrow head), consistent with cells entering the next cell cycle. *ddi1Δdsk2Δrad23Δ* cells separated their SPBs with the same kinetics as wild type cells, but then delayed or arrested with large buds and two SPBs (Figure 3C, red line). A very similar phenotype was seen in *dsk2Δrad23Δ* mutants (data not shown). Interestingly, the arrest observed in the *dsk2Δrad23Δ* and the triple deleted cells was not homogeneous, since some of the cells arrested as dumbbells with well separated SPBs, resembling anaphase cells (Figure 3B, right cells, and Figure 3D), while the rest of the arrested cells possessed SPBs that were separated by a distance typical of G2 cells (Figure 3B, arrow).

DAPI staining of DNA in these arrested cells confirmed that some cells were arrested in a G2-like stage with a single nucleus (Figure 3E, left two panels), while other cells were arrested in early (3rd panel) or late anaphase (rightmost panel). Arrest in G2 or anaphase could have been due to defects in spindle assembly. Therefore, to further explore these results and examine in more detail the dynamics of spindle assembly in the triple deletion, we constructed strains harboring a *TUB1:GFP* gene (Table 1). In agreement with the results observed in the *SPC42:GFP* strains, wild type cells and *ddi1Δdsk2Δrad23Δ* cells assembled their spindles with the same timing, relative to bud emergence (Figure 3F).

In a final series of experiments, we performed time courses in which cells were released from a nocodazole arrest at the restrictive temperature of 37°C (Figure 3G). The percentage of wild type cells with G2-SPBs (Figure 3G, top panel, blue line) began to decrease by 20 minutes after release from nocodazole and cells with G2-SPBs were completely depleted (i.e. the cells had undergone anaphase) 90 minutes after the release. In contrast, the triple deleted cells showed a delay in anaphase onset, as judged by the distance between the SPBs, and ~30% remained in G2 (Figure 3G, top panel, green line) at the time wild type G2-cells had been completely depleted. Similar data were obtained in strains expressing *TUB1-GFP*. Within about 10 minutes after release of the nocodazole block, almost all large budded cells had assembled G2 spindles (Figure 3G, lower panel). Wild type cells initiated spindle elongation ~20 minutes after release from nocodazole and by 40 minutes most of the cells had completed anaphase (Figure 3G, lower panel, blue line). In contrast, some *ddi1Δdsk2Δrad23Δ* cells remained in G2, as judged by their short (1–4 μM) spindles, even 90 minutes after the release (Figure 3G, lower panel, green line). Together, these results suggest *ddi1Δdsk2Δrad23Δ* cells delay cell cycle progression at two distinct points when grown at high temperatures: a fraction of the triple deleted cells delay before anaphase with duplicated SPBs but short spindles, whereas other cells delay in a mid-anaphase or late-anaphase state with partly-elongated or fully-elongated spindles. This suggests that combined deletion of *RAD23*, *DDI1* and *DSK2* affects spindle dynamics and cell cycle progression, but not SPB duplication.

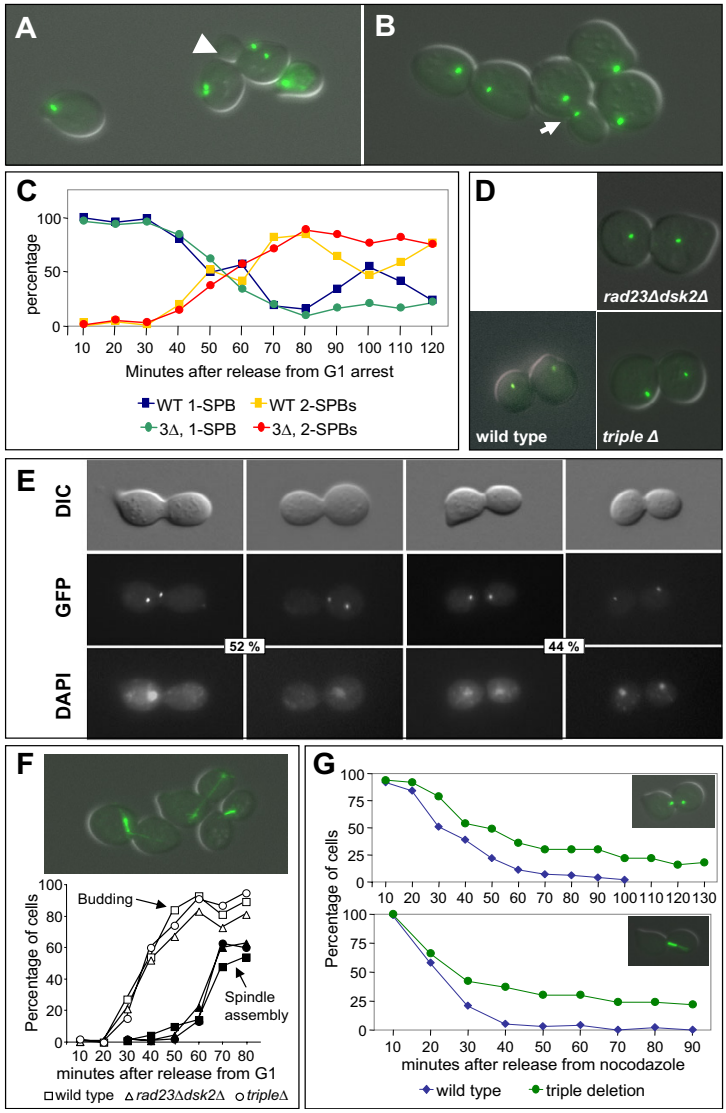

Figure 3

SPB duplication and spindle function. (A) Photomicrograph of wild type SPC42:GFP cells from time course in C, 110 minutes after alpha-factor release. Arrowhead shows rebudded cell that has already undergone a second SPB duplication. (B) Photomicrograph of *rad23Δdsk2Δddi1ΔSPC42:GFP* cells from time course in C, 110 minutes after alpha factor release. Arrow shows cell with G2-like short inter-SPB distance. (C) SPB duplication kinetics. Wild type (WT) and *rad23Δdsk2Δddi1Δ* (3Δ) cells were arrested with alpha factor and released into rich medium at 37°C. Samples were taken every 10 minutes and SPBs in each cell were counted. (D) Photomicrographs showing SPBs (*SPC42:GFP*) in wild type, *rad23Δdsk2Δ* and *rad23Δdsk2Δddi1Δ* cells at 90 minutes after release from G1 arrest at the restrictive temperature. (E) Photomicrographs showing DIC, *SPC42:GFP* signals, and DAPI staining of DNA in *rad23Δdsk2Δddi1Δ* cells at 180 minutes after release from G1 arrest at the restrictive temperature of 37°C. Left two panels show G2-like cells (52% of the large budded cells) with a single nucleus and SPBs typically separated by less than 4 μm. Right two panels show anaphase cells with divided nuclei (44% of the large budded cells) and SPBs typically separated by > 4 μm. The remaining large budded cells had stretched single nuclei. (F) Timing of bud emergence and spindle assembly after release from alpha-factor induced G1 arrest in wild type, *rad23Δdsk2Δ*, and *rad23Δdsk2Δddi1Δ* cells. Photomicrograph shows *rad23Δdsk2Δddi1ΔTUB1:GFP* cells at 37°C with both G2 and anaphase spindles. (G) Nocodazole release experiment in *rad23Δdsk2Δddi1ΔSPC42:GFP* cells. Cells were arrested with alpha factor, released into medium containing nocodazole and grown at 30°C for 2 hours, then washed and released into medium containing alpha factor to prevent rebudding upon progression to G1. Samples were taken every 10 minutes and SPBs with short G2-like inter-SPB distance (1–4 μm, inset micrograph) were counted (upper panel). This protocol was repeated in *rad23Δdsk2Δddi1Δ TUB1:GFP* cells (lower panel). Samples were taken every 10 minutes and short G2 spindles (inset micrograph) were counted.

Ddi1 homodimerization is necessary for pds1–128 *rescue*

In addition to their abilities to bind ubiquitin and the proteasome, Dsk2, Ddi1 and Rad23 share the ability to form homodimers [22,24]. The importance of dimerization for the function of these proteins is not known however. Dsk2 and Rad23 homodimerization involves their UBL and/or UBA domains [17,23,24], which makes it difficult to obtain mutants that disrupt homodimerization but remain capable of binding to ubiquitin and the proteasome. Ddi1, on the other hand does not require either the UBA or the UBL domains for homodimerization [22]. Therefore we tested whether homodimerization is important for the role of Ddi1 in one specific cell cycle related event; stabilization of Pds1–128. Firstly we more precisely defined the domain important for Ddi1 homodimerization to a region encompassing residues 184 to 285 (Figure 4A, plum). By deleting this region a new *DDI1* allele, *ddi1Δ184–285*, was obtained that was no longer able to dimerize with wild type Ddi1 but was still able to bind both Rad23 (Figure 4B) and ubiquitin (data not shown).

To ask whether Ddi1 dimerization was important for the role of Ddi1 in stabilizing Pds1–128, we overexpressed exogenous *ddi1Δ184–285* in a *pds1–128 DDI1* strain (Figure 4C) and asked whether this mutant could rescue the temperature sensitivity of this strain (Figure 4D). When overexpressed, neither *ddi1Δ184–285*, nor *ddi1* alleles that disrupt UBA-ubiquitin interaction (*ddi1ΔUBA* or *ddi1-L426A* [17]) rescued the temperature sensitivity of the *pds1–128* strain but overexpression of wild type *DDI1* or *ddi1-A407L* (a *ddi1*-UBA mutant that still interacts with ubiquitin) did rescue the temperature sensitivity of *pds1–128*. The protein levels of wild type *Ddi1* and the mutant forms after expression from the *GAL1* promoter were all similar (Figure 4C). This was also the case when tagged versions of *DDI1* and mutants were expressed from the *GAL1* promoter (Figure 2A). Therefore, these results suggest that both homodimerization and ubiquitin interaction are required for rescue of *pds1–128* by overproduced Ddi1.

Discussion

The UBL-UBA proteins Ddi1, Dsk2 and Rad23 are conserved from yeast to humans but are not essential for viability in yeast, suggesting that they might perform overlapping functions. We investigated this possibility by removing all three UBL-UBA proteins to find that they are essential for viability and cell cycle progression at high temperatures. Interestingly, these analyses did not provide evidence for a functional overlap between Ddi1 and Dsk2, but rather indicate that Rad23 has redundant functions with each of Ddi1 and Dsk2. A strain deleted for all three of these genes exhibited a synthetic effect, suggesting that *RAD23* has at least two independent roles that are required for viability at high temperatures; one of them shared with *DDI1* and the other one with *DSK2*. Interestingly, these redundancies do not seem to be a product of evolutionary divergence of duplicated genes, since neither *RAD23*, *DSK2* or *DDI1* are elements in the duplication blocks that have been described to have arisen after the yeast genome duplication event. Furthermore, they lie next to elements from different duplication blocks: block 28 for *RAD23*, block 46 for *DSK2* and block 13 for *DDI1* [35], which further suggests that they are functionally related, but not evolutionarily related.

Since these proteins have been shown to be involved in cell cycle related phenomena [28,31], we examined cell cycle progression at the restrictive temperature and found that *ddi1Δdsk2Δrad23Δ* cells arrest or delay in G2/M and anaphase. Surprisingly, the arrest observed in the triple deletion was not due to a failure in SPB duplication. We were also not able to observe SPB duplication defects in the *rad23Δdsk2Δ* double deletion strains at 37°C (data not shown), as had been previously reported [28]. We currently do not know the reason for this contradictory result, but it could be due to differences in the genetic background between the BF264-*15Daub* (this study) and S288c strains [28].

Interestingly, when we examined the kinetics of spindle elongation (Figure 3) we found that the arrest observed in the triple deletion strain was not homogeneous. There were two distinct populations: cells that arrested with G2-like spindles and had a single nucleus, and cells that arrested with partly or fully elongated spindles and divided nuclei. One possibility is that the *ddi1Δdsk2Δrad23Δ* cells progressively accumulate ubiquitinated substrates that somehow interfere with degradation of other cell cycle regulatory proteins. The existence of two arrest points might therefore be a reflection of two cell cycle stages at which proteasome function is required: some of the mutant cells may have accumulated enough substrates to enforce arrest at the earlier time point whereas other cells might be able to reach the latter cell cycle stage. However, the specific reasons why subpopulations of the triple deleted cells arrest at the earlier or later time point remain to be determined.

The ability of UBL-UBA proteins to both hetero and homodimerize, as well as to bind to polyubiquitin in tandem [23] suggests the possibility that UBL-UBA dimerization regulates binding of these proteins to ubiquitinated substrates, possibly by bringing more than one UBL-UBA protein in close proximity to the ubiquitin chain [23,36]. Ddi1 is the only UBL-UBA protein in which homodimerization does not involve the UBL or UBA domains, allowing the study of the role of dimerization without affecting its interaction with ubiquitin or the proteasome. We have shown that disruption of Ddi1 homodimerization affects

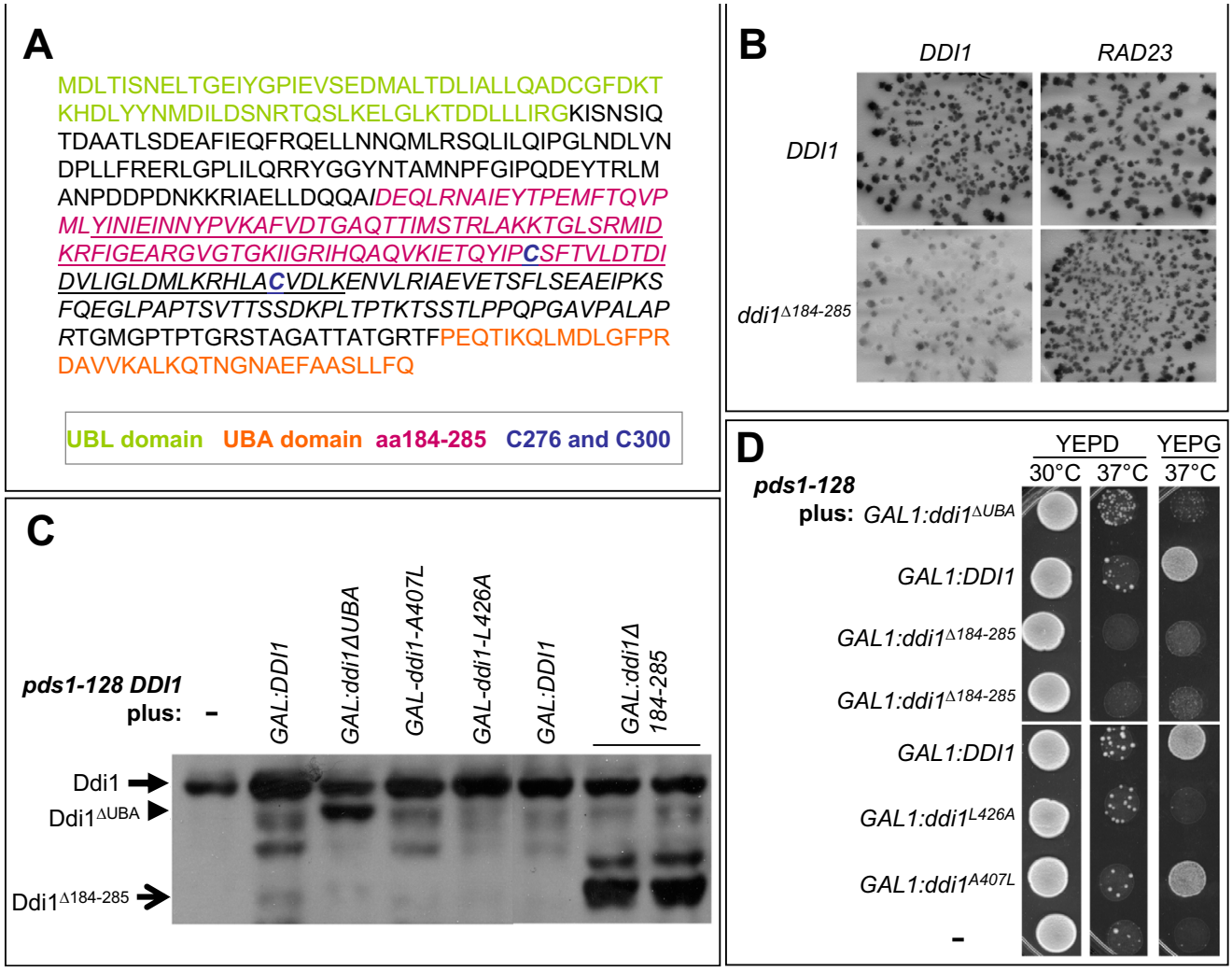

Figure 4

Ddi1 homodimerization is required for rescue of *pds1–128* temperature sensitivity. (A) Ddi1 sequence with the UBL and UBA domains in green and orange, respectively. Residues 184–285 are displayed in plum and C276 and C300 in blue. (B) Yeast two hybrid assay reveals that residues 184–285 are important for homodimerization of Ddi1 but not for its interaction with Rad23. Production of blue color in x-gal containing medium signals for a positive interaction between two proteins [17]. *ddi1Δ184–285* interaction with *DDI1* is highly reduced but *ddi1Δ184–285* interaction with *RAD23* is similar to that of wild type *DDI1*. (C) Western blots showing levels of different Ddi1 variants. Cells were grown on YEPR overnight, diluted 1:10 and expression of *DDI1* constructs was induced from the *GAL1* promoter by Galactose addition. These strains corresponded to the *pds1–128 DDI1* strains assayed in D and expressed the indicated constructs exogenously from *GAL1* in addition to expressing endogenous *DDI1* (which is approximately 47 kD) from its native locus. The leftmost lane contains a vector control, not expressing *DDI1* exogenously. The Western blot was probed with poly-clonal anti-sera against Ddi1 that recognized all of the mutant forms tested. Where wild type *DDI1* is expressed from the *GAL1* promoter, the upper band contains endogenous and exogenous Ddi1. (D) Rescue of *pds1–128* temperature sensitivity. Serial dilutions of the indicated strains (corresponding to those tested in D) were spotted onto YEPD or YEPG plates and incubated for 24 hr at the indicated temperatures.

Ddi1's ability to rescue the *pds1–128* temperature sensitivity, suggesting that Ddi1 homodimerization is necessary for its role in Pds1–128 stabilization. Ddi1 dimerization does not involve the cystein residues at positions 276 and 300 (Figure 4A, blue) since a C276S C300S double mutant is still able to interact with wild type Ddi1 (data not shown). Interestingly, it has been suggested that Pds1–128 stabilization by Ddi1 might be due to de-ubiquitination of Pds1–128 through the aspartyl-protease domain of Ddi1 (underlined in Figure 4A)[37]. Our results suggest that this could be possible. First, the *ddi1Δ184–285* allele lacks half of the aspartyl-protease

domain. Second, the aspartyl protease domain requires dimerization to become active, and the *ddi1Δ184–285* allele does not dimerize, suggesting that the predicted Ddi1 aspartyl-protease activity is not functional in *ddi1Δ184–285* and could hypothetically be involved in *pds1–128* rescue.

Conclusion

Redundancy of function among the budding yeast UBL-UBA proteins can be predicted based on their common abilities to bind ubiquitin and the proteasome. We have shown that the yeast UBL-UBA proteins have partially redundant functions, Rad23 being redundant with both Ddi1 and Dsk2. Surprisingly Ddi1 and Dsk2 do not share redundant functions but a synthetic phenotype is observed in the triple deletion strain in which cells become delayed in both G2 and anaphase.

Materials and methods

Strains and Plasmids

Strains are derivatives of BF264-*15Daub* [38], unless otherwise noted. Standard genetic procedures were used [39]. Spindles were visualized by expressing a *GFP:TUB1* construct [40,41] integrated at the *URA3* locus. SPBs were visualized by expressing a *GFP-SPC42* construct [28]. Cultures were grown on YEPD at 30°C unless otherwise stated. Micrographs were acquired with a Zeiss Axioplan2 Microscope, using an alpha-Plan-FLUAR 100X/1,45 oil objective, an Axiocam HRm camera and Axiovision software.

The *pB42AD-ddi1Δ184–285* vector was obtained by ClaI digestion of the *pB42AD-DDI1* two-hybrid vector [17,31] followed by self-ligation. *pYIPG2-DDI1* and *pYIG2-ddi1Δ184–285* were obtained by PCR from the respective pB42AD-vectors. The PCR fragments were cloned into a pCR2.1-TOPO vector (Invitrogen), cut out with BamHI and cloned into the pYIPG2 vector. These constructs were subsequently integrated at the *LEU2* locus. *pB42AD-DDI1-C276S-C300S* was constructed using the QuickChange site directed mutagenesis kit (Stratagene) using the following mutagenesis primers: DDIC300S-1 5'CTGAAAAGGCATTGGCTAGTGTGGACTTAAAGGAAAAC3', DDIC300S-2 5'GTTTTCCTTTAAGTCCACACTAGCCAAATGCCTTTTCAG3', DDIC276S-1 5'AAAATAGAAACACAATATATTCCAAGCAGTTTTACCGTCTTAGATACTG3' and DDIC276S-2 5'CAGTATCTAAGACGGTAAAACTGCTTGGAATATATTGTGTTTCTATTTT3'.

DSK2 was disrupted by homologous recombination using a fragment obtained by PCR from the FA6 cassette using the primers: dsk2kan-5' (5'-ATAAGACGGATCAAAGACACCGAATCATTCTAGCACGATACAGCTGAAGCTTCGTACGCT-3') and dsk2kan-3' (5'-TAGGGTAAAAGTATATAGGTTGCGGCATCTAGACGTTTATGCATAGGCCACTAGTGGATC-3')

Time courses

Yeast strains were grown in rich YEPD medium containing extra adenine overnight and diluted 1:20 before the experiment. For G1-release, diluted cultures were incubated for ~2 hours in the presence of 0.2 μg/mL alpha-factor (Sigma), then washed twice and released in fresh medium at 37°C. For nocodazole release, an alpha-factor arrest was performed first and cells were released into fresh medium at 37°C containing 15 μg/mL Nocodazole for 11/2 hours. Cells were washed twice with pre-warmed sterile water and released into pre-warmed rich medium containing 0.4 μg/mL alpha-factor to stop the cells from entering the next cell cycle. Alpha-factor was also added to the nocodazole-arrest medium 1/2 hour before releasing. At least 100 cells were counted per timepoint.

Western blots

Whole cell lysates were separated by SDS-PAGE (10% acrylamide w/v), transferred to an Immobilon PVDF membrane and probed using rabbit polyclonal anti-Ddi1 1:5000, or rabbit polyclonal anti-Rad23 1:5000 [22].

List of Abbreviations

GFP – Green Fluorescent Protein

SPB – Spindle Pole Body

UBA – Ubiquitin Associated

UBL – Ubiquitin Like

YEPD – Yeast Peptone Dextrose

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

Experiments performed by LADM, YK. Experiment design by KJW and DJC.

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